**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : | | (11) International Publication Number: |
|---|---|---|
| C12N 15/12, C07K 13/00<br>C12P 21/02, A61K 37/02<br>C07K 15/00 | A1 | WO 92/17583 |
| | | (43) International Publication Date: 15 October 1992 (15.10.92) |

(21) International Application Number: PCT/US91/02207

(22) International Filing Date: 29 March 1991 (29.03.91)

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
*With international search report.*
*With amended claims.*

(54) Title: ISOLATED VIRAL PROTEIN CYTOKINE ANTAGONISTS

(57) Abstract

Isolated viral proteins, and pharmaceutical compositions made therefrom, are disclosed which are capable of binding to cytokines, thereby functioning as cytokine antagonists. Also disclosed are processes for preparing isolated viral protein cytokine antagonists.

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# TITLE

Isolated Viral Protein Cytokine Antagonists

## BACKGROUND OF THE INVENTION

The present invention relates generally to the field of viral proteins, and more specifically to viral proteins having immunoregulatory activity.

Viruses are infectious particles which contain genetic elements that enable the virus to replicate within a living host cell. By sequencing the genes of viruses and analyzing the DNA sequence, it has been possible to identify many open reading frames (ORFs) comprising long stretches of triplet codons beginning with a translation-initiation codon (preceded by a ribosomal binding site) and uninterrupted by a translational stop codon. Most ORFs in viruses, however, have not been shown to code proteins. For example, the genomic organization and DNA sequence of several ORFs from the telomeric region of Shope fibroma virus (SFV) have recently been characterized (Upton et al.,*Virology 160*:20 (1987)). Although it has been shown that these ORFs are transcriptionally active and code for mRNAs, no proteins encoded by these mRNAs have yet been identified or isolated, nor has any biological function for the putative proteins (as surmised from the ORF) been identified. Similarly, the DNA sequence of telomeric region of the myxoma virus has been obtained and several ORFs identified; however, no protein encoded by these ORFs has been identified, isolated or characterized.

The present invention identifies a specific class of viral proteins having immunosuppressive activity, and provides a method for identifying and isolating such viral proteins. The invention also provides pharmaceutical compositions for regulating immune responses.

## SUMMARY OF THE INVENTION

The present invention provides isolated viral proteins having cytokine antagonist activity, and pharmaceutical compositions comprising such viral proteins for regulating immune responses. The present invention also provides processes for preparing isolated viral proteins having cytokine antagonist activity.

The isolated viral proteins of this invention are similar to cytokine binding proteins, such as the extracellular region of a cytokine receptor, and are capable of binding a cytokine and preventing the cytokine from binding to its receptor. The ability of such viral proteins to mimic the activity of a cytokine receptor (and thereby downregulate specific immune responses) enables the viral protein to circumvent the anti-viral defense mechanisms of the

host organisms and confers a selective advantage to the virus. The viral proteins of the present invention can be used to regulate immune responses in a therapeutic setting.

The present invention specifically provides isolated Shope fibroma virus (SFV) T2 protein, which is an expression product of the SFV T2 open reading frame, and isolated myxoma virus (MV) T2 protein, which is an expression product of the myxoma T2 open reading frame. Both SFV T2 protein and myxoma T2 protein have TNF antagonist activity.

These and other aspects of the present invention will become evident upon reference to the following detailed description of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

SEQ ID NO:1 and SEQ ID NO:2 depict the cDNA sequence and derived amino acid sequence of the Shope fibroma virus (SFV) T2 open reading frame (ORF). The SFV T2 ORF extends from nucleotide 1332 to 2306 and encodes an amino acid sequence designated as the c-phase reading frame.

SEQ ID NO:3 and SEQ ID NO:4 depict the cDNA sequence and derived amino acid sequence of the myxoma virus T2 ORF. The myxoma T2 ORF extends from nucleotide 2 to 979 and encodes an amino acid sequence designated as the b-phase reading frame.

## DETAILED DESCRIPTION OF THE INVENTION

The immune system protects the human body from infection and disease through the interaction of specialized white blood cells which recognize and destroy invading microbes and diseased cells. White blood cells, including T cells, B cells, granulocytes and macrophages, are controlled and coordinated by specific proteins known as cytokines, which direct the development, proliferation, function and effectiveness of these cells. Cytokines act upon immune cells by contacting and attaching (i.e., binding) specific proteins called cytokine receptors which are located on immune cell surfaces. The binding of a cytokine to its specific receptor initiates a complex series of events within the responsive cell which translates the cytokine's signal to that cell. This signal can then stimulate cell division or production of antibodies, enzymes or other cytokines, thereby controlling and coordinating the function of immune cells located throughout the body. In their native configuration, receptor proteins are present as intact human plasma membrane proteins having an extracellular region which binds to a ligand, a hydrophobic transmembrane region which causes the protein to be immobilized within the plasma membrane lipid bilayer, and a cytoplasmic or intracellular region which interacts with proteins and/or chemicals within the cell to deliver a biological signal to effector cells via a cascade of chemical reactions within the cytoplasm of the cell. The extracellular region thus

defines a domain of the receptor molecule to which a ligand can bind to transduce a biological signal.

The normal immune response can be weakened by overwhelming infection or other immunosuppressive conditions associated with the development of cancer. Immune system malfunction can also result in autoimmune diseases such as arthritis and diabetes, which result when a misdirected immune response destroys joint tissues or pancreatic cells. Transplant patients frequently suffer organ rejection, in which the immune system attacks the transplanted organ as a foreign body. In other immune disorders, the immune system overreacts to normal encounters with foreign substances, resulting in allergic conditions or asthma. Byproducts of severe immune responses can also be harmful, for example, in the inflammatory conditions know as cachexia and septic shock. Furthermore, cytokine-directed accumulation of white blood cells in response to infection can lead to inflammatory conditions which can exacerbate the severity of lung disease conditions such as emphysema.

Such misdirected or inappropriate immune responses may be counteracted by cytokine antagonists, which bind to the cytokine and prevent the cytokine from binding to its receptor, thereby inhibiting the initiation of an immune response.

The present invention relates to viral proteins which are capable of modulating the activity of cytokines by acting as cytokine antagonists. The viral proteins of the present invention have a sequence of amino acids which is similar to the ligand-binding region of a cytokine receptor (e.g., the extracellular region of the receptor) or to a soluble cytokine receptor and is capable of binding to the cytokine and preventing the cytokine from binding to its receptor.

Definitions

As used herein, the term "viral protein" refers to proteins encoded by RNA, DNA, mRNA or cDNA isolated or otherwise derived from a viral source.

"Isolated", as used in the context of the present invention to define the purity of viral proteins, refers to proteins which are substantially free of other human or viral proteins of natural or endogenous origin and contains less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics. Isolated viral proteins are detectable as a single protein band in a polyacrylamide gel by silver staining.

A "cytokine" is a specific protein which directs the development, proliferation, function and effectiveness of cells of the immune system. Specific examples of "cytokines" include, but are not limited to, the interleukins (e.g., IL-1, IL-2, IL-3, IL-4,

IL-5, IL-6, IL-7, IL-8), interferon (IFNα and IFN β), tumor necorsis factor (TNFα and TNF β) and various growth factors, such as GM-CSF, G-CSF, and CSF-1. Each of the above cytokines transduces a biological signal by binding to a receptor molecule specific to the cytokine.

A viral protein having "cytokine antagonist activity" inhibits, counteracts or neutralizes the biological activity of a cytokine. Cytokine antagonist activity may be effected by means of the viral protein sterically hindering the binding of a cytokine to its receptor, thereby preventing cytokine signal transduction. For example, a viral protein can sterically hinder the binding of a cytokine to its receptor by binding the cytokine or its receptor at or near a site required for cytokine/receptor binding. The viral protein thus physically prevents the cytokine and receptor from interacting and transducing a biological signal. Specific examples of viral proteins having cytokine antagonist activity include polypeptides encoded by the SFV T2 open reading frame and the myxoma virus T2 opening reading frame, designated herein as SFV T2 protein and myxoma virus T2 protein, respectively. The DNA sequence of the open reading frame encoding SFV T2 protein and the amino acid sequence of SFV T2 protein is set forth in SEQ ID NO:1. The DNA sequence of the open reading frame encoding myxoma T2 protein and the amino acid sequence of myxoma T2 protein is set forth in Figure 2.

SFV T2 and myxoma T2 are TNF antagonists. Tumor necrosis factor-α (TNFα, also known as cachectin) and tumor necrosis factor-β (TNFβ, also known as lymphotoxin) are homologous mammalian endogenous secretory proteins capable of inducing a wide variety of effects on a large number of cell types. The great similarities in the structural and functional characteristics of these two cytokines have resulted in their collective description as "TNF." Complementary DNA clones encoding TNFα (Pennica et al., *Nature 312*:724, 1984) and TNFβ (Gray et al., *Nature 312*:721, 1984) have been isolated.

TNF initiates its biological effect on cells by binding to specific TNF receptor protein expressed on the plasma membrane of a TNF-responsive cell. It is believed that TNFα and TNFβ share a common receptor. The amino acid sequences of SFV T2 and myxoma T2 are similar to the extracellular region of the receptor to which TNF binds, and mimic the TNF receptor by binding to TNF. SFV T2 and myxoma T2 thus inhibit binding of TNF to TNF receptor. Because of its ability to inhibit binding of TNF to TNF receptor, isolated SFV T2 and myxoma T2 protein compositions will be useful in diagnostic assays for TNF, as well as in raising antibodies to SFV T2 and myxoma T2 for use in diagnosis and therapy. In addition, purified SFV T2 and myxoma T2 compositions may be used directly in therapy to bind or scavenge TNF, thereby providing a means for regulating the immune activities of TNF. In order to study the structural and biological characteristics of SFV T2 and myxoma T2 and the roles played by SFV T2 and myxoma T2 in the responses

of various cell populations to viral infection by SFV and MV, or to use SFV T2 and myxoma T2 effectively in therapy, diagnosis, or assay, purified compositions of SFV T2 and myxoma T2 are needed. Such compositions, however, are obtainable in practical yields only by cloning and expressing genes encoding the receptors using recombinant DNA technology.

The terms "TNF receptor" and "TNF-R" refer to proteins having amino acid sequences of the native mammalian TNF receptor amino acid sequences.

A "soluble cytokine receptor", as used in the context of the present invention, refers to a protein, or a substantially equivalent analog, having an amino acid sequence corresponding to the extracellular region of a native cytokine receptor, for example polypeptides having the amino acid sequences substantially equivalent to the extracellular region of TNF receptor. Because soluble proteins are devoid of a transmembrane region, they are secreted from the host cell in which they are produced. Viral proteins having an amino acid sequence sufficiently similar to the extracellular region of a cytokine receptor or to a soluble cytokine receptor will retain the ability to bind the cytokine and inhibit the ability of the cytokine to transduce a signal via cell surface bound cytokine receptor proteins. When administered in therapeutic formulations, the viral proteins circulate in the body and bind to circulating cytokine molecules, preventing interaction of the cytokine with natural cytokine receptors and inhibiting transduction of cytokine-mediated biological signals, such as immune or inflammatory responses.

A viral protein has "cytokine antagonist activity" if the viral protein has a sequence of amio acids "sufficiently similar" to either the extracellular region of a cytokine receptor or to a soluble receptor that the viral protein is capable of inhibiting binding of the cytokine receptor to its ligand, thereby inhibiting cytokine signal transduction. Assays for determining cytokine binding inhibition are described below in Example 1. Inhibition of cytokine signal transduction can be determined by transfecting cells with recombinant cytokine receptor DNAs to obtain recombinant receptor expression. The cells are then contacted with the cytokine ligand and the resulting metabolic effects examined. If an effect results which is attributable to the action of the ligand, then the recombinant receptor has signal transducing activity. Exemplary procedures for determining whether a polypeptide has signal transducing activity are disclosed by Idzerda et al., *J. Exp. Med.* 171:861 (1990); Curtis et al., *Proc. Natl. Acad. Sci. USA 86*:3045 (1989); Prywes et al., *EMBO J. 5*:2179 (1986); and Chou et al., *J. Biol. Chem. 262*:1842 (1987). Alternatively, primary cells of cell lines which express an endogenous cytokine receptor and have a detectable biological response to the cytokine could also be utilized. Such procedures are used as controls for assaying inhibition of signal transduction by the viral protein cytokine antagonists of the present invention.

"Recombinant," as used herein, means that a protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a protein produced in a microbial expression system which is essentially free of native endogenous substances. Protein expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.

"Biologically active," as used throughout the specification as a characteristic of a cytokine or a cytokine receptor, means that a particular molecule shares sufficient amino acid sequence similarity with the cytokine or receptor to be capable of binding detectable quantities of the cytokine, or cross-reacting with anti-cytokine receptor antibodies raised against the cytokine from natural (i.e., nonrecombinant) sources.

"DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could also be used. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

The viral proteins of the present invention having cytokine antagonist activity are identified by isolating and then analyzing a viral protein, RNA, DNA, mRNA or cDNA to provide an amino acid sequence of the viral protein. The amino acid sequence of the viral protein is then compared with the amino acid sequence of a cytokine or cytokine receptor and those viral proteins are selectted and isolated which have a sequence of amino acids sufficiently similar to an extracellular region of a cytokine receptor or a soluble cytokine receptor that the viral protein is capable of inhibiting binding of the cytokine receptor to its ligand. Alternatively, viral proteins can be selected and isolated which have a sequence similar to a cytokine and are capable of binding to a cytokine receptor (without transducing a cytokine signal) and inhibiting binding of the cytokine to its receptor.

Alternative methods for identifying viral proteins having cytokine antagonist activity include selecting a viral RNA, DNA, mRNA or cDNA capable of hybridization under moderately stringent conditions (50°C, 2x SSC) to DNA or cDNA clones encoding a cytokine binding protein and isolating the protein. DNA or RNA sequences capable of

hybridization to DNA clones encoding a cytokine binding protein under such conditions would be expected to be sufficiently similar to the cytokine binding protein to be capable of binding to the cytokine and inhibiting binding of the cytokine to its receptor.

Proteins and Analogs

The present invention provides isolated proteins having cytokine antagonist activity. Such proteins are substantially free of contaminating endogenous materials and, optionally, without associated native-pattern glycosylation. Derivatives of the viral proteins within the scope of the invention also include various structural forms of the primary protein which retain biological activity. Due to the presence of ionizable amino and carboxyl groups, for example, a protein may be in the form of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives are prepared by linking particular functional groups to amino acid side chains or at the N- or C-termini. Other derivatives of the protein within the scope of this invention include covalent or aggregative conjugates of the protein or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast α-factor leader). Protein fusions can comprise peptides added to facilitate purification or identification of viral proteins (e.g., poly-His). The amino acid sequence of the viral proteins can also be linked to the peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK) (Hopp et al., *Bio/Technology* 6:1204,1988.) The latter sequence is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*.

Protein derivatives may also be used as immunogens, reagents in receptor-based immunoassays, or as binding agents for affinity purification procedures of cytokines or other binding ligands. Viral protein derivatives may also be obtained by cross-linking agents, such as M-maleimidobenzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and lysine residues. Proteins may also be covalently bound through reactive side

groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-activated, carbonyldiimidazole-activated or tosyl-activated agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, proteins may be used to selectively bind (for purposes of assay or purification) antibodies raised against the viral protein or against cytokine receptors which are similar to the viral protein.

The present invention also includes viral proteins with or without associated native-pattern glycosylation. Proteins expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of viral DNAs in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of viral protein having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn-$A_1$-Z, where $A_1$ is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between $A_1$ and Z, or an amino acid other than Asn between Asn and $A_1$.

Viral protein derivatives may also be obtained by mutations of the native viral proteins or its subunits. A viral protein mutant, as referred to herein, is a polypeptide homologous to a viral protein but which has an amino acid sequence different from the native viral protein because of a deletion, insertion or substitution.

Bioequivalent analogs of viral proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those having physicochemical characteristics resembling those of the residue to be replaced. Similarly, when a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered. Subunits of viral proteins may be constructed by deleting terminal or internal residues or sequences.

Mutations in nucleotide sequences constructed for expression of analog viral proteins must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the receptor mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed viral protein mutants screened for the desired activity.

Not all mutations in the nucleotide sequence which encodes a viral protein will be expressed in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A, incorporated herein by reference), or to provide codons that are more readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene 42*:133, 1986); Bauer et al. (*Gene 37*:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462 disclose suitable techniques, and are incorporated by reference herein.

Expression of Recombinant Viral Protein Cytokine Antagonists

The proteins of the present invention are preferably produced by recombinant DNA methods by inserting a DNA sequences encoding viral protein into a recombinant expression vector and expressing the DNA sequence in a recombinant microbial expression system under conditions promoting expression.

DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being inserted in a recombinant expression vector and expressed in a recombinant transcriptional unit.

Recombinant expression vectors include synthetic or cDNA-derived DNA fragments encoding viral proteins or bioequivalent analogs operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation, as described in detail below. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame.

DNA sequences encoding viral proteins which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Other embodiments include sequences capable of hybridizing under moderately stringent conditions (50°C, 2x SSC) to the DNA sequences encoding viral proteins, and other sequences which are degenerate to those which encode the viral proteins.

Transformed host cells are cells which have been transformed or transfected with expression vectors constructed using recombinant DNA techniques and which contain sequences encoding the viral proteins of the present invention. Transformed host cells may express the desired viral protein, but host cells transformed for purposes of cloning or amplifying viral DNA do not need to express the viral protein. Expressed viral proteins will preferably be secreted into the culture supernatant, depending on the DNA selected, but may be deposited in the cell membrane. Suitable host cells for expression of viral proteins include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce viral proteins using RNAs derived from the DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*,

Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

Prokaryotic expression hosts may be used for expression of viral proteins that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the β-lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful bacterial expression system employs the phage λ $P_L$ promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ $P_L$ promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Recombinant viral proteins may also be expressed in yeast hosts, preferably from the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will generally contain an origin of replication from the 2μ yeast plasmid or an autonomously replicating sequence (ARS), promoter, DNA encoding the viral protein, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g.,

the ampicillin resistance gene of *E. coli* and *S. cerevisiae* trp1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of a structural sequence downstream. The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pBR322 for selection and replication in *E. coli* ($Amp^r$ gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α-factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem. 258*:2674, 1982) and Beier et al. (*Nature 300*:724, 1982). The yeast α-factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. *See, e.g.*, Kurjan et al., *Cell 30*:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984. The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978, selecting for $Trp^+$ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4°C prior to further purification.

Various mammalian or insect cell culture systems can be employed to express recombinant protein. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *BioITechnology 6*:47 (1988). Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney

cells, described by Gluzman (*Cell 23*:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *BglI* site located in the viral origin of replication is included. Further, viral genomic promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol. 23*:935, 1986).

A particularly preferred eukaryotic vector for expression of viral protein DNA is disclosed below in Examples 2 and 6. This vector, referred to as pCAV/NOT, was derived from the mammalian high expression vector pDC201 and contains regulatory sequences from SV40, adenovirus-2, and human cytomegalovirus.

Purified viral proteins or analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts.

For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. For

example, a suitable affinity matrix can comprise a viral protein or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a viral protein composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant viral protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant viral protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express viral protein as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog. 296*:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Viral protein synthesized in recombinant culture is characterized by the presence of non-viral cell components, including proteins, in amounts and of a character which depend upon the purification steps taken to recover the viral protein from the culture. These components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of viral protein free of other proteins which may be normally associated with the viral protein as it is found in nature in its species of origin, e.g. in cells, cell exudates or body fluids.

Administration of Viral Protein Compositions

The present invention provides methods of using therapeutic compositions comprising an effective amount of a viral protein and a suitable diluent and carrier, and

methods for regulating an immune response. The use of SFV T2 and myxoma T2 proteins in conjuction with soluble cytokine receptors, e.g., TNF receptor, is also contemplated.

For therapeutic use, purified viral protein is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, SFV T2 and myxoma T2 protein compositions administered to suppress immune function can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a therapeutic agent will be administered in the form of a composition comprising purified protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the viral protein with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in trials. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth.

SFV T2 and myxoma T2 proteins are administered for the purpose of inhibiting TNF dependent responses. TNF is used clinically as an antitumor agent and results in severe toxicities. The toxicities associated with the administration of TNF are identical to the effects that the cytokine manifests when it is produced in normal or overactive immune responses. It is believed that TNF produced as a result of the immune response to malignant tissue is a causative factor of cachexia. In addition, TNF is produced in the course of other immune reactions such as the body's response to severe bacterial infection where TNF production can contribute to the devlopment of septic shock. The production of other key cytokines (IL-1, IL-2 or a number of colony stimulating factors) can also induce significant host production of TNF. Thus, the side effects of these cytokines at certain doses administered to human patients have been attributed to the induction of TNF production. Because TNF binds to a specific TNF receptor present on the surface of responseive cells, viral TNF antagonists, such as SFV T2 and myxoma T2 may be useful as a therapy for cachexia or septic shock or to treat side effects associated with cytokine therapy.

The following examples are offered by way of illustration, and not by way of limitation.

# EXAMPLES

## Example 1

### Binding Assays

A. *Radiolabeling of TNFα and TNFβ.* Radiolabeled TNFα and TNFβ (used in various assays for TNF antagonists) was derived as follows. Recombinant human TNFα, in the form of a fusion protein containing a hydrophilic octapeptide at the N-terminus, was expressed in yeast as a secreted protein and purified by affinity chromatography (Hopp et al., *Bio/Technology 6*:1204, 1988). Purified recombinant human TNFβ was purchased from R&D Systems (Minneapolis, MN). Both proteins were radiolabeled to a specific activity of 2 x $10^{15}$ cpm/mmole using the commercially available solid phase agent, Iodogen (Pierce). In this procedure, 5 μg of Iodogen was plated at the bottom of a 10 X 75 mm glass tube and incubated for 20 minutes at 4°C with 75 μl of 0.1 M sodium phosphate, pH 7.4 and 20 μl (2 mCi) Na $^{125}$I. This solution was then transferred to a second glass tube containing 5 μg TNFα (or TNFβ) in 45 μl PBS for 20 minutes at 4°C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex G-25 (Sigma) equilibrated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) sodium azide and 20 mM Hepes pH 7.4 (binding medium). The final pool of $^{125}$I-TNF was diluted to a working stock solution of 1 x $10^{-7}$ M in binding medium and stored for up to 3 weeks at 4°C without significant loss of receptor binding activity.

B. *Detection of SFV T2 Binding to TNF Receptors.* Two separate binding assays were used to measure T2 protein binding to TNF receptors. In the first method, the presence of SFV T2 in COS-7 cell supernatants was measured by inhibition of $^{125}$I-TNFα binding to U937 cells. Supernatants from COS cells transfected with recombinant SFV T2 ORF constructs were harvested three days post-transfection. Serial two-fold dilutions of this supernatant were pre-incubated with 0.3 nM $^{125}$I-TNFα (specific activity 1 x $10^{15}$ cpm/mmole) for two hours at 4°C prior to the addition of 2 x $10^6$ U937 cells. The cells are incubated for an additional two hours at 4°C, after which free and cell bound human $^{125}$I-TNFα were separated using a pthalate oil separation method (Dower et al., *J. Immunol. 132*:751, 1984) essentially as described by Park et al. (*J. Biol. Chem. 261*:4177, 1986). Non-specific ligand binding in all assays was determined by the inclusion of a 200 molar excess of unlabeled ligand.

In the second method, $^{125}$I-TNF binding to T2 protein was detected directly by nitrocellulose dot blots. The ability of TNF receptor or T2 to be stably adsorbed to nitrocellulose from detergent extracts of human cells yet retain binding activity provided a

means of detecting T2. Cell extracts were prepared by mixing a cell pellet with a 2 x volume of PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (2 mM phenylmethyl sulfonyl fluoride, 10 μM pepstatin, 10 μM leupeptin, 2 mM o-phenanthroline and 2 mM EGTA) by vigorous vortexing. The mixture was incubated on ice for 30 minutes after which it was centrifuged at 12,000 x g for 15 minutes at 8°C to remove nuclei and other debris. Alternatively, recombinant T2 protein in the form of COS supernatants were mixed with an equal volume of PBS/1% Triton X-100 and a cocktail of the same protease inhibitors. Two microliter aliquots of cell extracts or T2 protein extracts were placed on dry BA85/21 nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and allowed to dry. The membranes were incubated in tissue culture dishes for 4 hours in Tris (0.05 M) buffered saline (0.15 M) pH 7.5 containing 3% w/v BSA to block nonspecific binding sites. The membrane was then covered with $5 \times 10^{-11}$ M $^{125}$I-TNF in PBS + 3% BSA and incubated for 2 hr at 4°C with shaking. At the end of this time, the membranes were washed 3 times in ice-cold PBS, dried and placed on Kodak X-Omat AR film for 18 hr at -70°C.

## Example 2

Expression of the SFV T2 ORF

A vector (pKTH-1) containing the Shope Fibroma Virus T2 opening reading frame (SFV T2 ORF) cloned into pUC19 was obtained from Dr. Grant McFadden of the University of Alberta, Edmonton, Canada. A SpeI/BamHI restriction fragment containing a majority the SFV T2 open reading frame was excised from pKTH-1 by digesting with SpeI and BamHI restriction enzymes, resulting in a partial SFV T2 ORF cDNA fragment from which had been deleted the first 7 codons (including the ATG initiation codon) of the 5' terminus. The 5' terminal coding sequence was reconstructed by ligating to the partial SFV cDNA fragment the following synthetic oligonucleotide, which incorporated a consensus sequence for optimum translation initiation and contained a 5' terminus compatible with an Asp718 restriction site:

Asp718 SpeI

```
GTACCGCCACCATGCTTCGTTTAATTGCACTA
    GCGGTGGTACGAAGCAAATTAACGTGATGATC
```

The resulting cDNA was ligated into the eukaryotic expression vector pDC302 which was digested with the Asp718 and BglII restriction enzymes. pDC302 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20842, USA, under the name pCAV/NOT-IL-7R, Accession Number 68014. The resulting

expression vector was designated pDC302-SFVT2ORF. pDC302 was assembled from pDC201 (described by Sims et al., *Science 241*:585, 1988 and derived from pMLSV, described by Cosman et al., *Nature 312*: 768, 1984), SV40 and cytomegalovirus DNA and comprises, in sequence with the direction of transcription from the origin of replication: (1) SV40 sequences from coordinates 5171-270 including the origin of replication, enhancer sequences and early and late promoters; (2) cytomegalovirus sequences including the promoter and enhancer regions (nucleotides 671 to +63 from the sequence published by Boechart et al. (*Cell 41*:521, 1985); (3) adenovirus-2 sequences containing the first exon and part of the intron between the first and second exons of the tripartite leader, the second exon and part of the third exon of the tripartite leader and a multiple cloning site (MCS) containing sites for XhoI, KpnI, SmaI, NotI and *BglI*; (4) SV40 sequences from coordinates 4127-4100 and 2770-2533 that include the polyadenylation and termination signals for early transcription; (5) sequences derived from pBR322 and virus-associated sequences VAI and VAII of pDC201, with adenovirus sequences 10532-11156 containing the VAI and VAII genes, followed by pBR322 sequences from 4363-2486 and 1094-375 containing the ampicillin resistance gene and origin of replication.

SFV T2 protein was then transiently expressed in monkey COS-7 cells as follows. A subconfluent layer COS-7 cells was transfected with pDC302-SFVT2ORF using DEAE-dextran followed by choroquine treatment, as described by Luthman et al., *Nucl. Acids Res. 11*:1295 (1983) and McCutchan et al., *J. Natl. Cancer Inst. 41*:351 (1968). The cells were then grown in culture for three days to permit transient expression of the inserted SFV T2 ORF sequences. After three days, cell culture supernatants and the cell monolayers were assayed as described in Example 1, and TNF binding and TNF/TNF receptor binding inhibition was confirmed. COS cells are then bulked up in sufficient quantities to yield several liters of conditioned medium containing microgram quantities of SFV T2 protein.

## Example 3

Purification of SFV T2 Protein by TNF Affinity Chromatography

SFV T2 protein is purified from COS cell supernatants of Example 2 using TNF as an affinity ligand. To obtain large amounts of recombinant TNF for preparation of a TNF affinity matrix, a Flag®-TNF fusion protein containing the "Flag®" octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys fused to the amino terminus of TNF was constructed and expressed. This octapeptide sequence does not alter the biological activity of TNF, is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling facile purification of the expressed TNF (Hopp et al., *BioITechnology 6*:1204 (1988).

The Flag®-TNF fusion protein is coupled to Affigel-10 (Bio-Rad) or CnBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Inc.) according to the manufacturer's suggestions and as previosuly described by Urdal et al., *J. Biol. Chem. 263*:2870 (1988). COS cell conditioned medium from Example 2 is harvested and centrifuged and the resulting conditioned medium (RPMI 1640) is adjusted to 1% BSA, 0.1% sodium azide, 20 mM HEPES, pH 7.4.. To the conditioned medium is added a cocktail of protease inhibitors (2mM PMSF, 2 mM O-phenanthroline, 1 mM pepstatin, 1 mM leupeptin). The resulting medium is applied to a Flag®-TNF affinity column equilibrated with PBS, pH 7.4. The column is then washed with 10 column volumes of PBS, pH 7.4, after which bound protein is eluted with 0.1 M glycine-HCl, pH 3.0. Eluate containing SFV T2 protein is immediately neutralized with 80 ml of 1.0 M HEPES, pH 7.4 and aliquots removed for binding assays (described in Example 1, above) and analysis by SDS-PAGE as previously described by Urdal, *J. Biol. Chem. 263*:2870 (1988).

## Example 4

Purification of SFV T2 Protein Using Reversed-Phase HPLC

SFV T2 protein is also purified by conventional methods using Flag®-TNF binding as a biological assay for detection of SFV T2 activity. Flag®-TNF is produced as described in Example 3 above. COS cell conditioned medium from Example 2 is harvested and centrifuged and the resulting conditioned medium (RPMI 1640) is adjusted to 1% BSA, 0.1% sodium azide, 0.5 M $CaCl_2$ and 20 mM HEPES, pH 7.4.. To the conditioned medium is added a cocktail of protease inhibitors (2mM PMSF, 2 mM O-phenanthroline, 1 mM pepstatin, 1 mM leupeptin). SFV T2 protein is purifed from the resulting medium by conventional purification methods, including ion-exchange, hydrophobic interaction, gel exclusion and refersed-phase HPLC.

## Example 5

Preparation of Monoclonal Antibodies to SFV T2 Protein

Preparations of purified recombinant SFV T2, for example, or transfected COS cells expressing high levels of SFV T2 are employed to generate monoclonal antibodies against SFV T2 using conventional techniques, for example, those disclosed in U.S. Patent 4,411,993. Such antibodies are likely to be useful in interfering with TNF binding to TNF receptors, for example, in ameliorating toxic or other undesired effects of TNF, or as components of diagnostic or research assays for TNF or soluble TNF receptor.

To immunize mice, SFV T2 immunogen is emulsified in complete Freund's adjuvant and injected in amounts ranging from 10-100 µg subcutaneously into Balb/c mice.

Ten to twelve days later, the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1. Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a HAT selective medium (hypoxanthine, aminopterin, and thymidine) to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with SFV T2 or TNF receptor, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in U.S. Patent 4,703,004. Positive clones are then injected into the peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high concentrations (>1 mg/ml) of anti-SFV T2 monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

## Example 6

Expression of the Myxoma Virus T2 ORF

A vector (pMTN-6) containing the Myxoma Virus T2 opening reading frame (MYXOMA T2 ORF) was obtained from Dr. Grant McFadden of the University of Alberta, Edmonton, Canada. This vector was constructed by inserting a Myxoma BamHI fragment (see Russell & Robbins, *Virology 90*:147) into the BamHI site of pUC19. A NlaIII fragment containing the entire coding region of the MYXOMA T2 ORF was isolated from pMyBT-5 and cloned into the SphI site of pMH21p to create pMTN-6.

The MYXOMA T2 ORF was excised from pMTN-6 by digesting with HindIII and PstI restriction enzymes, resulting in a complete MYXOMA T2 ORF cDNA fragment. The resulting cDNA was blunt-ended and ligated into the eukaryotic expression vector pDC302 which was digested with the SmaI restriction enzyme. The resulting expression vector was designated pDC302-MVT2ORF-1.

SFV T2 protein was then transiently expressed in monkey COS-7 cells as follows. A subconfluent layer COS-7 cells was transfected with pDC302-MVT2ORF using DEAE-dextran followed by choroquine treatment, as described by Luthman et al., *Nucl. Acids Res. 11*:1295 (1983) and McCutchan et al., *J. Natl. Cancer Inst. 41*:351 (1968). The cells were then grown in culture for three days to permit transient expression of the inserted

MYXOMA T2 ORF sequences. After three days, cell culture supernatants and the cell monolayers are assayed as described in Example 1. The cell culture supernatants did not inhibit binding of TNF to TNF-receptor, possibly because the HindIII/PstI restriction fragment did not contain specific sequences 5' of the coding region which are required for expression. Accordingly, myxoma T2 ORF cloned into the mammalian expression vector pDC302 utilizing the polymerase chain reaction (PCR) technique. This method inserts a CACC nucleotide sequence upstream of the initiation codon which is important for optimum initiation of translation (Kozak, *Mol. Cell. Bio.* 8:2737 (1988)). The following primers are used in this construction:

5' End Primer

5'-CCTTGCGGCCGCCACCATGTTTCGTTTAACGCTACTACT-3'
NotI site Initiation Codon

3' End Primer

5'CCTTAGATCTGTAATCTATGAAACGAGTCTACAT-3'
BglII site

The PCR product thus contains NotI and BglII restriction sites at the 5' and 3' termini, respectively. These restriction sites are used to clone into pDC302. The template for the PCR reaction is the clone myxoma T2 clone, described above, which contains the myxoma T2 ORF (pMTN-6). The DNA sequences encoding the myxoma T2 ORF (including the upstream Kozak sequences) are then amplified by PCR, substantially as described by Innis et al., eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, 1990). The resulting amplified clone is then isolated at ligated into pDC302 and transiently expressed in monkey COS-7 cells as described above. COS cells are then bulked up in sufficient quantities to yield several liters of conditioned medium containing microgram quantities of SFV T2 protein.

## Example 7

### Purification of Myxoma T2 Protein by TNF Affinity Chromatography

Myxoma T2 protein is purified from COS cell supernatants of Example 6 using TNF as an affinity ligand. To obtain large amounts of recombinant TNF for preparation of a TNF affinity matrix, a Flag®-TNF fusion protein containing the "Flag®" octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys fused to the amino terminus of TNF was constructed and expressed. This octapeptide sequence does not alter the biological activity of TNF, is highly antigenic and provides an epitope reversibly bound by a specific monoclonal

antibody, enabling facile purification of the expressed TNF (Hopp et al., *Bio/Technology* 6:1204 (1988).

The Flag®-TNF fusion protein is coupled to Affigel-10 (Bio-Rad) or CnBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Inc.) according to the manufacturer's suggestions and as previosuly described by Urdal et al., *J. Biol. Chem.* 263:2870 (1988). COS cell conditioned medium from Example 6 is harvested and centrifuged and the resulting conditioned medium (RPMI 1640) is adjusted to 1% BSA, 0.1% sodium azide, 20 mM HEPES, pH 7.4.. To the conditioned medium is added a cocktail of protease inhibitors (2mM PMSF, 2 mM O-phenanthroline, 1 mM pepstatin, 1 mM leupeptin). The resulting medium is applied to a Flag®-TNF affinity column equilibrated with PBS, pH 7.4. The column is then washed with 10 column volumes of PBS, pH 7.4, after which bound protein is eluted with 0.1 M glycine-HCl, pH 3.0. Eluate containing myxoma T2 protein is immediately neutralized with 80 ml of 1.0 M HEPES, pH 7.4 and aliquots removed for binding assays (described in Example 1, above) and analysis by SDS-PAGE as previously described by Urdal, *J. Biol. Chem.* 263:2870 (1988).

## Example 8

Purification of SFV T2 or Myxoma T2 Protein Using Reversed-Phase HPLC

Myxoma T2 protein is also purified by conventional methods using Flag®-TNF binding as a biological assay for detection of myxoma T2 activity. Flag®-TNF is produced as described in Example 3 above. COS cell conditioned medium from Example 6 is harvested and centrifuged and the resulting conditioned medium (RPMI 1640) is adjusted to 1% BSA, 0.1% sodium azide, 0.5 M $CaCl_2$ and 20 mM HEPES, pH 7.4. To the conditioned medium is added a cocktail of protease inhibitors (2mM PMSF, 2 mM O-phenanthroline, 1 mM pepstatin, 1 mM leupeptin). Myxoma T2 protein is purifed from the resulting medium by conventional purification methods, including ion-exchange, hydrophobic interaction, gel exclusion and refersed-phase HPLC.

## Example 9

Preparation of Monoclonal Antibodies to Mycoma T2 Protein

Preparations of purified recombinant myxoma T2, for example, or transfected COS cells expressing high levels of myxoma T2 are employed to generate monoclonal antibodies against myxoma T2 using conventional techniques, for example, those disclosed in U.S. Patent 4,411,993. Such antibodies are likely to be useful in interfering with TNF binding to TNF receptors, for example, in ameliorating toxic or other undesired effects of TNF, or as components of diagnostic or research assays for TNF or soluble TNF receptor.

To immunize mice, myxoma T2 immunogen is emulsified in complete Freund's adjuvant and injected in amounts ranging from 10-100 µg subcutaneously into Balb/c mice. Ten to twelve days later, the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1. Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a HAT selective medium (hypoxanthine, aminopterin, and thymidine) to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with myxoma T2 or TNF receptor, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in U.S. Patent 4,703,004. Positive clones are then injected into the peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high concentrations (>1 mg/ml) of anti-myxoma T2 monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Goodwin, Raymond G.

(ii) TITLE OF INVENTION: Isolated Viral Protein Cytokine Antagonists

(iii) NUMBER OF SEQUENCES: 4

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rabbit fibroma virus

(vii) IMMEDIATE SOURCE:
(B) CLONE: T2 ORF

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 192..1169

(D) OTHER INFORMATION:
(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 192..1166
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GTGATTGAGT TGTTCATGAG GTTGATCGCG GATTATGAGA TTTCACATAT CAGGTCCGTG      60

ATCAATATTC GTTTACAATG CTTCGCTCCT CGAAAAAGTC GTAACATCTA AATTGGCTCT     120

CTCATTCGGT TATAATTGAT TCCTTCTTTT TCTTGTACAA AAATATAAAA ATAATTACAA     180

CATTATATAT T ATG CTT CGT TTA ATT GCA CTA CTA GTA TGT GTC GTG TAC       230
             Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr
               1               5                  10

GTA TAC GGA GAT GAT GTA CCG TAT TCT TCC AAT CAA GGA AAA TGT GGA       278
Val Tyr Gly Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly
     15                  20                  25

GGA CAC GAC TAC GAA AAA GAC GGA CTG TGT TGT GCA TCC TGT CAT CCA       326
Gly His Asp Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro
 30                  35                  40                  45

GGG TTT TAT GCC TCT AGA TTG TGC GGA CCC GGG TCC AAT ACG GTG TGT       374
Gly Phe Tyr Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys
                 50                  55                  60

TCT CCG TGT GAA GAC GGA ACC TTT ACG GCG AGT ACT AAC CAT GCC CCT       422
Ser Pro Cys Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro
             65                  70                  75

GCG TGC GTA AGT TGT CGA GGT CCG TGT ACG GGG CAT CTA TCC GAG TCT       470
Ala Cys Val Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser
         80                  85                  90

CAA CCG TGC GAT AGA ACC CAC GAT AGA GTC TGC AAT TGT TCT ACG GGG       518
Gln Pro Cys Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly
     95                  100                 105

AAC TAT TGT CTG TTG AAA GGA CAG AAC GGA TGT AGG ATA TGT GCC CCC       566
Asn Tyr Cys Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro
110                 115                 120                 125

CAG ACA AAG TGT CCC GCG GGA TAT GGC GTC TCT GGA CAC ACG CGA GCG       614
Gln Thr Lys Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala
                130                 135                 140

GGA GAT ACT CTC TGT GAG AAA TGT CCT CCG CAT ACG TAT TCC GAT TCT       662
Gly Asp Thr Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser
            145                 150                 155

CTG TCT CCA ACA GAG AGA TGC GGT ACA TCG TTT AAT TAC ATC AGT GTG       710
Leu Ser Pro Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val
        160                 165                 170

GGA TTC AAT CTA TAT CCC GTA AAC GAA ACG TCT TGT ACG ACG ACC GCT       758
Gly Phe Asn Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala
    175                 180                 185
```

```
GGA CAC AAC GAA GTG ATC AAA ACG AAG GAG TTT ACA GTT ACG TTA AAT      806
Gly His Asn Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn
190                 195                 200                 205

TAC ACG GAT TGT GAT CCT GTC TTT CAC ACG GAA TAC TAC GCA ACG AGT      854
Tyr Thr Asp Cys Asp Pro Val Phe His Thr Glu Tyr Tyr Ala Thr Ser
                210                 215                 220

GGA AAA GAA GGA GCT GGT GGA TTC TTC ACG GGA ACA GAT ATA TAC CAG      902
Gly Lys Glu Gly Ala Gly Gly Phe Phe Thr Gly Thr Asp Ile Tyr Gln
            225                 230                 235

AAC ACC ACC AAG GTG TGT ACA CTC AAC GTG GAG ATC CAG TGT TCT GAG      950
Asn Thr Thr Lys Val Cys Thr Leu Asn Val Glu Ile Gln Cys Ser Glu
        240                 245                 250

GGA GAC GAT ATA CAT ACA TTG CAG AAG ACG AAC GGG GGG TCT ACC ATG      998
Gly Asp Asp Ile His Thr Leu Gln Lys Thr Asn Gly Gly Ser Thr Met
    255                 260                 265

CCT CAT TCG GAG ACG ATT ACC GTC GTA GGA AGT TGT CTG TCC GAC GTT     1046
Pro His Ser Glu Thr Ile Thr Val Val Gly Ser Cys Leu Ser Asp Val
270                 275                 280                 285

AAT GTA GAT ATC ATG TAC AGC GAC ACC AAC CAC CCC GGG GAG GTC GAT     1094
Asn Val Asp Ile Met Tyr Ser Asp Thr Asn His Pro Gly Glu Val Asp
                290                 295                 300

GAC TTC GTG GAA TAT CAT TGG GGG ACG CGT CTC CGT TTC TTT CCC TTA     1142
Asp Phe Val Glu Tyr His Trp Gly Thr Arg Leu Arg Phe Phe Pro Leu
            305                 310                 315

CCC AAA CGA TGT ACC CCA GTC TCG TAG GGTTTTTCTT TCTCGTTAAT          1189
Pro Lys Arg Cys Thr Pro Val Ser  .
        320                 325

TTCTTAAAAA A                                                       1200
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 325 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Leu Arg Leu Ile Ala Leu Leu Val Cys Val Val Tyr Val Tyr Gly
 1               5                  10                  15

Asp Asp Val Pro Tyr Ser Ser Asn Gln Gly Lys Cys Gly Gly His Asp
            20                  25                  30

Tyr Glu Lys Asp Gly Leu Cys Cys Ala Ser Cys His Pro Gly Phe Tyr
        35                  40                  45

Ala Ser Arg Leu Cys Gly Pro Gly Ser Asn Thr Val Cys Ser Pro Cys
    50                  55                  60
```

```
Glu Asp Gly Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val
 65                  70                  75                  80

Ser Cys Arg Gly Pro Cys Thr Gly His Leu Ser Glu Ser Gln Pro Cys
                 85                  90                  95

Asp Arg Thr His Asp Arg Val Cys Asn Cys Ser Thr Gly Asn Tyr Cys
            100                 105                 110

Leu Leu Lys Gly Gln Asn Gly Cys Arg Ile Cys Ala Pro Gln Thr Lys
        115                 120                 125

Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Ala Gly Asp Thr
    130                 135                 140

Leu Cys Glu Lys Cys Pro Pro His Thr Tyr Ser Asp Ser Leu Ser Pro
145                 150                 155                 160

Thr Glu Arg Cys Gly Thr Ser Phe Asn Tyr Ile Ser Val Gly Phe Asn
                165                 170                 175

Leu Tyr Pro Val Asn Glu Thr Ser Cys Thr Thr Thr Ala Gly His Asn
            180                 185                 190

Glu Val Ile Lys Thr Lys Glu Phe Thr Val Thr Leu Asn Tyr Thr Asp
        195                 200                 205

Cys Asp Pro Val Phe His Thr Glu Tyr Tyr Ala Thr Ser Gly Lys Glu
    210                 215                 220

Gly Ala Gly Gly Phe Phe Thr Gly Thr Asp Ile Tyr Gln Asn Thr Thr
225                 230                 235                 240

Lys Val Cys Thr Leu Asn Val Glu Ile Gln Cys Ser Glu Gly Asp Asp
                245                 250                 255

Ile His Thr Leu Gln Lys Thr Asn Gly Gly Ser Thr Met Pro His Ser
            260                 265                 270

Glu Thr Ile Thr Val Val Gly Ser Cys Leu Ser Asp Val Asn Val Asp
        275                 280                 285

Ile Met Tyr Ser Asp Thr Asn His Pro Gly Glu Val Asp Asp Phe Val
    290                 295                 300

Glu Tyr His Trp Gly Thr Arg Leu Arg Phe Phe Pro Leu Pro Lys Arg
305                 310                 315                 320

Cys Thr Pro Val Ser
                325
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1064 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Myxoma virus

(vii) IMMEDIATE SOURCE:
(B) CLONE: T2 ORF

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 2..979
(D) OTHER INFORMATION:

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..982
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
C ATG TTT CGT TTA ACG CTA CTA CTC GCG TAC GTC GCG TGC GTA TAC          46
  Met Phe Arg Leu Thr Leu Leu Leu Ala Tyr Val Ala Cys Val Tyr
    1               5                  10                  15

GGG GGC GGT GCC CCG TAT GGC GCG GAT CGA GGA AAA TGT AGA GGG AAC        94
Gly Gly Gly Ala Pro Tyr Gly Ala Asp Arg Gly Lys Cys Arg Gly Asn
                20                  25                  30

GAC TAC GAA AAG GAC GGA CTG TGT TGT ACC TCC TGT CCT CCC GGG TCG       142
Asp Tyr Glu Lys Asp Gly Leu Cys Cys Thr Ser Cys Pro Pro Gly Ser
            35                  40                  45

TAC GCC TCT AGG TTA TGC GGA CCC GGG TCC GAC ACG GTA TGT TCT CCG       190
Tyr Ala Ser Arg Leu Cys Gly Pro Gly Ser Asp Thr Val Cys Ser Pro
        50                  55                  60

TGC AAG AAC GAA ACC TTT ACG GCG AGT ACG AAC CAC GCT CCC GCG TGC       238
Cys Lys Asn Glu Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys
    65                  70                  75

GTA AGT TGT CGA GGG CGG TGC ACA GGC CAC CTA TCC GAG TCT CAA TCG       286
Val Ser Cys Arg Gly Arg Cys Thr Gly His Leu Ser Glu Ser Gln Ser
80                  85                  90                  95

TGT GAT AAA ACC CGC GAT AGA GTC TGC GAC TGT TCT GCG GGG AAC TAT       334
Cys Asp Lys Thr Arg Asp Arg Val Cys Asp Cys Ser Ala Gly Asn Tyr
                100                 105                 110

TGT CTG TTG AAA GGA CAG GAG GGG TGT AGG ATA TGC GCT CCC AAA ACG       382
Cys Leu Leu Lys Gly Gln Glu Gly Cys Arg Ile Cys Ala Pro Lys Thr
            115                 120                 125

AAG TGT CCC GCG GGG TAT GGC GTC TCC GGA CAT ACG CGT ACG GGC GAC       430
Lys Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Thr Gly Asp
        130                 135                 140

GTG CTC TGC ACA AAA TGT CCT CGG TAC ACG TAT TCC GAC GCC GTA TCC       478
Val Leu Cys Thr Lys Cys Pro Arg Tyr Thr Tyr Ser Asp Ala Val Ser
    145                 150                 155

TCC ACG GAG ACG TGT ACC TCG TCG TTT AAC TAC ATC AGC GTG GAA TTC       526
Ser Thr Glu Thr Cys Thr Ser Ser Phe Asn Tyr Ile Ser Val Glu Phe
160                 165                 170                 175
```

```
AAC CTA TAT CCC GTA AAC GAC ACG TCT TGT ACG ACG ACC GCC GGA CCC        574
Asn Leu Tyr Pro Val Asn Asp Thr Ser Cys Thr Thr Thr Ala Gly Pro
                180                 185                 190

AAC GAA GTG GTT AAA ACG TCG GAG TTC TCG GTT ACG CTA AAT CAC ACG        622
Asn Glu Val Val Lys Thr Ser Glu Phe Ser Val Thr Leu Asn His Thr
            195                 200                 205

GAT TGT GAT CCC GTC TTC CAC ACG GAA TAC TAC GGA ACG AGC GGC AGC        670
Asp Cys Asp Pro Val Phe His Thr Glu Tyr Tyr Gly Thr Ser Gly Ser
        210                 215                 220

GAG GGC GCG GGA GGA TTC TTC ACC GGG ATG GAT AGG TAC CAG AAT ACG        718
Glu Gly Ala Gly Gly Phe Phe Thr Gly Met Asp Arg Tyr Gln Asn Thr
    225                 230                 235

ACC AAA ATG TGT ACG CTT AAT ATA GAG ATA CGG TGC GTC GAG GGA GAC        766
Thr Lys Met Cys Thr Leu Asn Ile Glu Ile Arg Cys Val Glu Gly Asp
240                 245                 250                 255

GCC GTG CGT ACT ATA CCG AGG ACG AGC GAC GGG GTC GGC GTC CTA TCT        814
Ala Val Arg Thr Ile Pro Arg Thr Ser Asp Gly Val Gly Val Leu Ser
                260                 265                 270

CAT TCG GAA ACG ATT ACC GTG ATA GGA GGG TGC CTG TCC GAC GTG AAC        862
His Ser Glu Thr Ile Thr Val Ile Gly Gly Cys Leu Ser Asp Val Asn
            275                 280                 285

GTA GAT ATC GAG TAC AGC GAC AGT AAT CAT CCC GAG GAG GTC GAC GAC        910
Val Asp Ile Glu Tyr Ser Asp Ser Asn His Pro Glu Glu Val Asp Asp
        290                 295                 300

TTC GTG GAA TAC CAT TGG GGT ACA CGC CTC CGT CTC TTT CCC TCA CCC        958
Phe Val Glu Tyr His Trp Gly Thr Arg Leu Arg Leu Phe Pro Ser Pro
    305                 310                 315

AAA CGA TGT AGA CTC GTT TCA TAG ATTACGGATT TTCTTCTAGT TAAATTCTTA      1012
Lys Arg Cys Arg Leu Val Ser  .
320                 325

AAAAAAAGTC GAATTATAAT AAAACGTGGG CGTATAGAAG AACTCTATCA TG             1064
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Phe Arg Leu Thr Leu Leu Leu Ala Tyr Val Ala Cys Val Tyr Gly
 1               5                  10                  15

Gly Gly Ala Pro Tyr Gly Ala Asp Arg Gly Lys Cys Arg Gly Asn Asp
            20                  25                  30

Tyr Glu Lys Asp Gly Leu Cys Cys Thr Ser Cys Pro Pro Gly Ser Tyr
        35                  40                  45
```

```
Ala Ser Arg Leu Cys Gly Pro Gly Ser Asp Thr Val Cys Ser Pro Cys
     50                  55                  60

Lys Asn Glu Thr Phe Thr Ala Ser Thr Asn His Ala Pro Ala Cys Val
 65                  70                  75                  80

Ser Cys Arg Gly Arg Cys Thr Gly His Leu Ser Glu Ser Gln Ser Cys
                 85                  90                  95

Asp Lys Thr Arg Asp Arg Val Cys Asp Cys Ser Ala Gly Asn Tyr Cys
            100                 105                 110

Leu Leu Lys Gly Gln Glu Gly Cys Arg Ile Cys Ala Pro Lys Thr Lys
        115                 120                 125

Cys Pro Ala Gly Tyr Gly Val Ser Gly His Thr Arg Thr Gly Asp Val
    130                 135                 140

Leu Cys Thr Lys Cys Pro Arg Tyr Thr Tyr Ser Asp Ala Val Ser Ser
145                 150                 155                 160

Thr Glu Thr Cys Thr Ser Ser Phe Asn Tyr Ile Ser Val Glu Phe Asn
                165                 170                 175

Leu Tyr Pro Val Asn Asp Thr Ser Cys Thr Thr Thr Ala Gly Pro Asn
            180                 185                 190

Glu Val Val Lys Thr Ser Glu Phe Ser Val Thr Leu Asn His Thr Asp
        195                 200                 205

Cys Asp Pro Val Phe His Thr Glu Tyr Tyr Gly Thr Ser Gly Ser Glu
    210                 215                 220

Gly Ala Gly Gly Phe Phe Thr Gly Met Asp Arg Tyr Gln Asn Thr Thr
225                 230                 235                 240

Lys Met Cys Thr Leu Asn Ile Glu Ile Arg Cys Val Glu Gly Asp Ala
                245                 250                 255

Val Arg Thr Ile Pro Arg Thr Ser Asp Gly Val Gly Val Leu Ser His
            260                 265                 270

Ser Glu Thr Ile Thr Val Ile Gly Gly Cys Leu Ser Asp Val Asn Val
        275                 280                 285

Asp Ile Glu Tyr Ser Asp Ser Asn His Pro Glu Glu Val Asp Asp Phe
    290                 295                 300

Val Glu Tyr His Trp Gly Thr Arg Leu Arg Leu Phe Pro Ser Pro Lys
305                 310                 315                 320

Arg Cys Arg Leu Val Ser
                325
```

## CLAIMS

We claim:

1. An isolated viral protein having cytokine antagonist activity.

2. An isolated viral protein according to claim 1, wherein the viral protein has a sequence of amino acids sufficiently similar to an extracellular region of a cytokine receptor that the viral protein is capable of binding to the cytokine and preventing the cytokine from binding to its receptor.

3. An isolated viral protein according to claim 2, wherein the viral protein has a sequence of amino acids sufficiently similar to a soluble cytokine receptor that the viral protein is capable of binding to the cytokine and preventing the cytokine from binding to its receptor.

4. An isolated viral protein according to claim 1, wherein the protein has TNF antagonist activity.

5. An isolated viral protein according to claim 4, wherein the protein has an amino acid sequence sufficiently similar to an extracellular region of TNF receptor that the viral protein is capable of binding to TNF and preventing TNF from binding to TNF receptor.

6. An isolated viral protein according to claim 1, wherein the DNA encoding the viral protein is capable of hybridization to a DNA sequence encoding an extracellular region of a cytokine receptor under moderately stringent conditions (50°C, 2X SSC).

7. An isolated viral protein acording to claim 6, wherein the cytokine receptor is TNF receptor.

8. An isolated viral protein according to claim 1, wherein the viral protein is Shope fibroma virus T2 protein.

9. An isolated protein according to claim 8, having the sequence of amino acids encoded by the sequence of nucleotides 192-1166 of SEQ ID NO:1.

10. An isolated viral protein according to claim 1, wherein the viral protein is myxoma virus T2 protein.

11. An isolated protein according to claim 10, having the sequence of amino acids encoded by the sequence of nucleotides 2-979 of SEQ ID NO:3.

12. A pharmaceutical composition for regulating an immune response, comprising an effective amount of a viral protein according to claim 1, and a suitable diluent or carrier.

13. A pharmaceutical composition for regulating an immune response, comprising an effective amount of a viral protein according to claim 2, and a suitable diluent or carrier.

14. A pharmaceutical composition for regulating an immune response, comprising an effective amount of a viral protein according to claim 3, and a suitable diluent or carrier.

15. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 4, and a suitable diluent or carrier.

16. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 5, and a suitable diluent or carrier.

17. A pharmaceutical composition for regulating an immune response, comprising an effective amount of a viral protein according to claim 6, and a suitable diluent or carrier.

18. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 7, and a suitable diluent or carrier.

19. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 8, and a suitable diluent or carrier.

20. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 9, and a suitable diluent or carrier.

21. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 10, and a suitable diluent or carrier.

22. A pharmaceutical composition for regulating a TNF mediated immune response, comprising an effective amount of a viral protein according to claim 11, and a suitable diluent or carrier.

23. A process for preparing an isolated viral protein having cytokine antagonist activity, comprising:

(a) analyzing a viral protein, RNA, DNA , mRNA, or cDNA to provide an amino acid sequence of the viral protein; and

(b) selecting and isolating a viral protein having a sequence of amino acids sufficiently similar to an extracellular region of a cytokine receptor that the viral protein is capable of binding to the cytokine and preventing the cytokine from binding to its receptor.

24. A process for preparing an isolated viral protein according to claim 23, wherein the viral protein has an amino acid sequence sufficiently similar to an extracellular region of TNF receptor that the viral protein possess TNF antagonist activity.

25. A process for preparing an isolated viral protein having cytokine antagonist activity, comprising:

(a) isolating a viral protein, RNA, DNA, mRNA, or cDNA complementary to viral RNA;

(b) analyzing the viral protein, RNA, DNA , mRNA, or cDNA to provide an amino acid sequence of the viral protein; and

(c) selecting and isolating a viral protein having an amino acid sequence sufficiently similar to an extracellular region of a cytokine receptor that the viral protein is capable of binding to the cytokine and preventing the cytokine form binding to its receptor.

26. A process for preparing an isolated viral protein according to claim 25, wherein the viral protein has an amino acid sequence sufficiently similar to the extracellular region of TNF receptor that the viral protein is capable of binding to TNF and preventing TNF from binding to TNF receptor.

27. A process for preparing an isolated viral protein having cytokine antagonist activity according to claim 1, comprising:

(a) selecting a viral RNA, DNA, mRNA, or cDNA capable of hybridization under moderately stringent conditions (50°C, 2X SSC) to DNA or cDNA clones encoding a cytokine binding protein; and

(b) isolating the viral protein.

28. A process for preparing an isolated viral protein according to claim 27, wherein the viral protein has an amino acid sequence sufficiently similar to the extracellular region of TNF receptor that the viral protein is capable of binding to TNF and preventing TNF from binding to TNF receptor.

AMENDED CLAIMS

[received by the International Bureau
on 27 March 1992 (27.03.92);
original claims 1-28 replaced by amended claims 1-6 (1 page)]

1. An isolated viral protein having TNF antagonist activity selected from the group consisting of Shope fibroma virus T2 protein and myxoma virus T2 protein.

2. An isolated protein according to claim 1, comprising the sequence of amino acids 1-325 of SEQ ID NO:1.

3. An isolated protein according to claim 1, comprising the sequence of amino acids 1-326 of SEQ ID NO:3.

4. An isolated viral protein, wherein the DNA encoding the viral protein is capable of hybridization to the sequence of nucleotides 192-1166 of SEQ ID NO:1 or nucleotides 2-979 of SEQ ID NO:3 under moderately stringent conditions (50°C, 2X SSC).

5. A pharmaceutical composition for regulating an immune response, comprising an effective amount of a viral protein according to claims 1-5, and a suitable diluent or carrier.

6. A process for preparing an isolated viral protein having cytokine antagonist activity according to claim 1, comprising:

(a) selecting a viral RNA, DNA, mRNA, or cDNA capable of hybridization under moderately stringent conditions (50°C, 2X SSC) to DNA or cDNA clones encoding Shope fibroma T2 viral protein or myxoma virus T2 viral proteins; and

(b) selecting clones that encode a viral protein capable of binding to TNF and preventing TNF from binding to TNF receptors; and

(c) isolating the viral protein.

# INTERNATIONAL SEARCH REPORT

International Application . PCT/US 91/02207

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C07K13/00; C12P21/02; A61K37/02
C07K15/00

**II. FIELDS SEARCHED**

**Minimum Documentation Searched**[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C07K |

**Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched**[8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**[9]

| Category * | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| P,X | BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 176, no. 1, 15 April 1991, DULUTH, MINNESOTA US pages 335 - 342; Smith, Craig A.; Davis, Terri; Wignall, Janis M.; Din, Wenie S.; Farrah, Theresa; Upton, C.; McFadden, G.; Goodwin, Raymond G.: 'T2 open reading frame from the shope fibroma virus encodes a soluble form of the TNF receptor' see the whole document --- -/-- | 1-9, 12-21, 23-28 |

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step .

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 13 JANUARY 1992 | 27. 01. 92 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | NAUCHE S.A. |

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| X | VIROLOGY<br>vol. 160, no. 1, 1987,<br>pages 20 - 30;<br>Upton, C.; DeLange, A. M.; McFadden, G.:<br>'Tumorigenic poxviruses: genomic organization and DNA sequence of the telomeric region of the shope fibroma virus genome.'<br>cited in the application | 1-9 |
| Y | see the whole document | 12-21, 23-28 |
| | --- | |
| Y | EP,A,0 418 014 (IMMUNEX CORPORATION) 20 March 1991<br>see page 1 - page 12, line 1-40; claims 1-20 | 12-21, 23-28 |
| | --- | |
| A | SCIENCE.<br>vol. 248, 25 May 1990, LANCASTER, PA US<br>pages 1019 - 1022;<br>Smith, Craig A. et al.: 'A receptor for tumor necrosis factor defines an unusual familiy of cellular and viral proteins.'<br>see the whole document | 1-28 |
| | --- | |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9102207 SA 51931

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 13/01/92

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A-0418014 | 20-03-91 | AU-A- 6178190 | 08-04-91 |
| | | JP-A- 3133382 | 06-06-91 |
| | | WO-A- 9103553 | 21-03-91 |